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Emerging perspectives in the research of bovine babesiosis and anaplasmosis

Carlos E. Suarez*, Susan Noh

Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, WA 99164-6630, USA

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ABSTRACT

The *Babesia bovis* and *B. bigemina* apicomplexan protozoa in conjunction with the rickettsia *Anaplasma marginale* are intraerythrocytic pathogens that are responsible for the most prevalent and costly tick borne diseases (TBD's) of cattle worldwide. These organisms are historically associated as they can cause clinically related hemolytic diseases in cattle, are all transmitted by *Rhiphicephallus* (*Boophilus*) ticks, and share an uncanny ability to evade the immune systems of the vertebrate hosts, causing persistent disease. In addition, acute babesiosis and anaplasmosis can be prevented quite effectively by combining tick control and vaccination with living attenuated organisms. However these methods of control have numerous limitations and improved approaches are needed. Importantly, immunizations of cattle with inactivated experimental *Babesia* and *Anaplasma* vaccines can elicit variable degrees of protection, indicating the feasibility for the development of inactivated or subunit vaccines. A new research toolbox that includes full genome sequencing combined with the improved ability to genetically modify the organisms is enhancing our understanding of their biology. An emerging paradigm is the use of recently developed *Babesia* and *Anaplasma* transfection methods for functional gene characterizations and for vaccine development. Promising recently identified subunit vaccine candidates are also emerging, including babesial proteases, putative rhoptry, microneme, and sexual stage antigens, as well as subdominant, conserved, *A. marginale* outer membrane major surface proteins. However, significant knowledge gaps on the role of key parasite molecules involved in cell invasion, adhesion, asexual and sexual reproduction, tick transmission, and evasion of the immune system, remain. A better understanding of the biology of these organisms and the protective immune responses will positively contribute toward the goal of developing improved immunological and pharmacological interventions against these elusive pathogens that are responsible for the most devastating TBD's of cattle. Importantly, the currently available research toolbox provides basic research instruments for helping close current knowledge gaps which will aid the design and production of effective vaccines and alternative pharmacological interventions.

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1. Introduction

Tickborne diseases (TBD) are important factors limiting the development of livestock industries worldwide. In particular, *Babesia* parasites and *Anaplasma* rickettsia, are responsible for important diseases of large economic, social, and epidemiological impact (De Vos, 1992; De Castro, 1997; Bock et al., 2004; Nari, 1995).

Although *Babesia* parasites are apicomplexan protozoa and *Anaplasma marginale* is a rickettsia, they were historically linked since they form part of a complex of diseases also known as cattle tick fever, sharing important features:

* Corresponding author. Tel.: +1 509 335 6341; fax: +1 509 335 8328.
E-mail address: ces@vetmed.wsu.edu (C.E. Suarez).

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Table 1
Babesia bovis and *B. bigemina* antigens currently investigated for the development of novel methods of control of bovine babesiosis.

Molecule/parasite	Putative function – features	Stage-localization	References
RAP-1, *B. bovis*, RAP-1 a,b,c *B. bigemina*	Erythrocyte binding, invasion–neutralization sensitive; signal peptide; immunodominant; conserved; surface exposed epitopes	Blood and tick stages: rhoptry–merozoites and sporozoites; surface; punctate pattern	Suarez et al. (1991, 1993, 1998, 2003), Dalrymple et al. (1993), Yokoyama et al. (2006) and Mosqueda et al. (2002a,b)
BboRhop68, *B. bovis*	Post-invasion function, egression? Trans-membrane domains. Immunogenic	Blood stages: intra-erythrocytic merozoites, trophozoites	Baravalle et al. (2010)
RAP-1 related antigen (RRA) *B. bovis*	Rhoptry? Invasion? Signal peptide, neutralization sensitive. Subdominant- lowly expressed	Rhoptry? Merozoites	Suarez et al. (2011) and Brayton et al. (2007)
VMSA: MSA-1, MSA-2, a, b, c of *B. bovis* Gp45 of *B. bigemina*	Erythrocyte recognition and attachment? Neutralization sensitive; signal peptide; immunodominant; variable (MSA2c more conserved); GPI anchor; glycosilated	Blood and tick stages: merozoites; sporozoites Surface exposed; merozoites; sporozoites	Hines et al. (1989, 1992, 1995a,b) Suarez et al. (2000), Jasmer et al. (1992a), Florin-Christensen et al. (2002) and Fisher et al. (2001)
Bb-AMA-1; BbTRAP *B. bovis*, Bb-AMA-1 *B. bigemina*	Invasion? Neutralization sensitive, conserved. Immunogenic? Signal peptide-transmembrane domains	Micronemes? Secreted?	Gaffar et al. (2003a,b) and Torina et al. (2010)
Spherical body proteins SBP-1, 2, 3, 4	Parasitosphorous vacuole? Food acquisition? Immunogenic	Spherical body; erythrocyte cytoplasm; Merozoites	Hines et al. (1995a,b), Dowling et al. (1996), Jasmer et al. (1992b) and Terkawi et al. (2011)
Bbo-MIC-1 *B. bovis*	Cell recognition, attachment and invasion? Neutralization sensitive; sialic acid binding domains; conserved. Immunogenic	Blood stages. Micronemes? Merozoites	Silva et al. (2010)
Bbo-6cys A, B, C, D, E, F *B. bovis*	Sexual stage development? Neutralization sensitive; conserved; immunogenic	Bbo-6cysF: blood stages. Sexual stage parasites?	Silva et al. (2011)
Large multigene families (vesa, smorfs). *B. bovis*	Persistent infection, evasion, antigenic variation; infected erythrocyte sequestration. Immunogenic	Blood stages: surface of infected erythrocytes (vesa), and unknown (smorfs)	Allred (2001), Allred and Al-Khedery (2006), Al-Khedery and Allred (2006) and Brayton et al. (2007)
Bov57. *B. bovis*, *T* (*B*) *equi*, *B. bigemina*	Syntenic to protective *T. parva* antigen p67, conserved	Blood and tick stages: merozoites, sporozoites	Freeman et al. (2010)
Bovipain-2, *B. bovis*; *B. bigemina*?	Conserved, immunogenic; Nutrition?	Blood stages: cytoplasm of *B. bovis* and of infected erythrocytes	Mesplet et al. (2010)

they both infect exclusively erythrocytes in the vertebrate hosts causing a clinically related acute disease, and they are transmitted by *Rhiphicephallus* (*Boophilus*) ticks, often co-occurring in endemic areas. Control of these TBD's worldwide is currently based in large part on the use of live vaccines that are in some cases trivalent (based in *B. bovis*, *B. bigemina* attenuated strains and *Anaplasma marginale* subspecies *centrale* organisms), and on the use of acaricides targeting their tick vectors (De Castro, 1997; De Vos, 1992; Standfast et al., 2003; Benavides et al., 2000; Vidotto et al., 1998). In addition, both *Babesia* and *Anaplasma* can cause persistent infections by avoiding the bovine immune system using antigenic variation (Barbet, 2009).

Babesiosis and anaplasmosis remain prevalent worldwide, in part, because the current approaches available for control have many important limitations, and need to be improved. Significant factors currently affecting the control of babesiosis and anaplasmosis include increased resistance to acaricides by ticks (Rosario-Cruz et al., 2009; Jonsson et al., 2008), and the numerous drawbacks of the current live vaccines (De Waal and Combrink, 2006; De Vos and Bock, 2000; Kocan et al., 2010). Whole genome sequencing of *Babesia* and *Anaplasma* organisms together with the development of novel research tools allowing genetic transformation are providing new insights on their genetics (Brayton et al., 2005, 2007; Felsheim et al., 2010; Herndon et al., 2010; Suarez and McElwain, 2010). Yet, despite of these advances, the most important limitations for developing improved control tools against these are the numerous and significant gaps in our understanding of the biology of *Babesia* and *Anaplasma*, and in the protective immune responses that they can elicit in cattle.

In this review we briefly summarize our current understanding of bovine babesiosis caused by *B. bovis* and *B. bigemina*, and anaplasmosis, describe some current gaps in knowledge, and highlight some new perspectives emerging from recent advances in research.

2. Bovine babesiosis

Cattle babesiosis is caused mainly by the tick-borne apicomplexan parasites *B. bovis*, *B. bigemina,* and *B. divergens* (which is prevalent in Europe and has zoonotic potential). The geographic distribution of *Babesia* para-

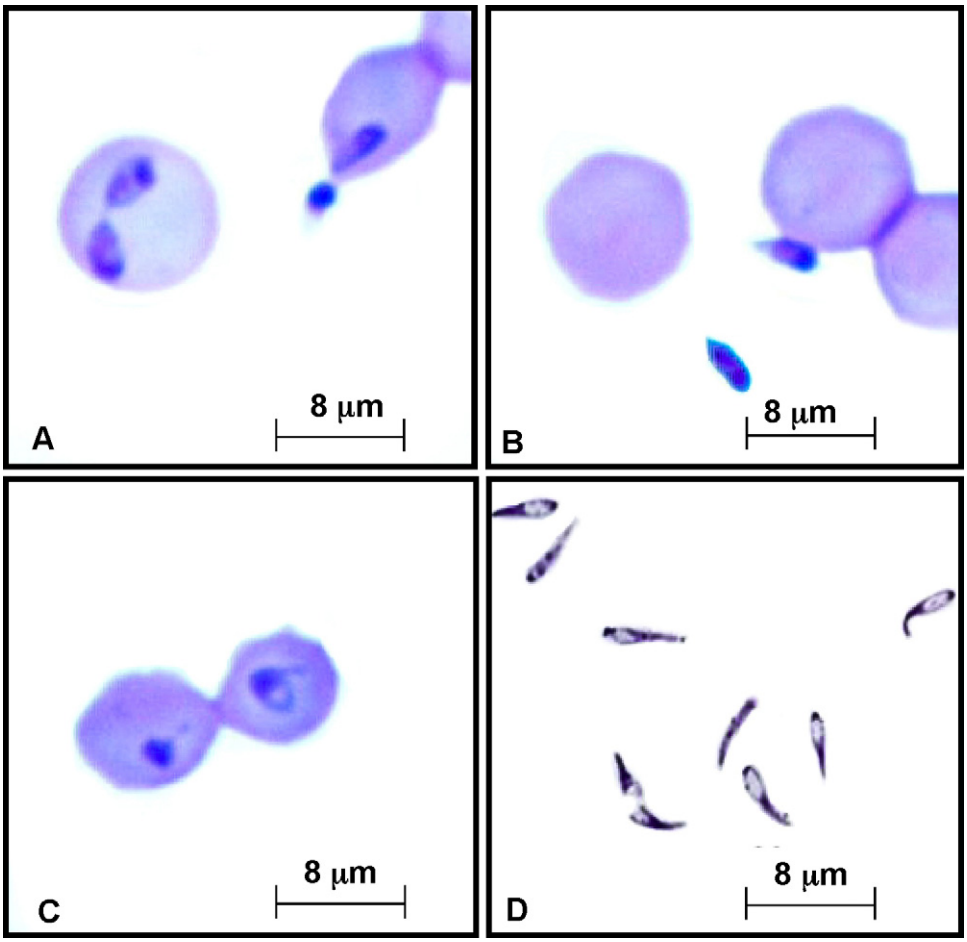

Fig. 1. Blood stages during the development of *Babesia bovis* parasites in bovine erythrocyte *in vitro* cultures: (A) a mature intracellular *B. bovis* merozoite pair is shown on the left side of the Panel. (B) *B. bovis* free merozoite, and a free merozoite attaching to a bovine erythrocyte surface; (C) intracellular *B. bovis* trophozoites; (D) *B. bovis* kinetes isolated from the hemolymph of *Riphicephalus microplus* infected with *B. bovis*.

sites corresponds with that of their tick vectors (Graham and Hourrigan, 1977), mainly *Rhiphicephalus* (*Boophilus*) *microplus* and *R* (*B*) *annulatus* in tropical and subtropical regions worldwide (Chauvin et al., 2009). In addition, other *Babesia* parasites of limited geographic distribution, *B. ovata* and *B. major*, are also known to cause disease in cattle. Because *B. bovis* and *B. bigemina* are the most significant species infecting cattle and, they will be the main focus of this review. Babesiosis caused by *B. divergens* was reviewed in deep elsewhere (Zintl et al., 2003). Cattle babesiosis is an acute disease that becomes persistent in surviving animals (Goff et al., 2001; Trueman and Blight, 1978). The most common clinical symptoms associated with acute disease include fever, hemolytic anemia, anorexia, lethargy, hemoglobinuria, tachycardia, and icterus. *B. bovis* is known for causing more severe disease often resulting in cerebral babesiosis, characterized by convulsions, hyperaesthesia, and paralysis, concomitant with sequestration of parasites in brain capillaries. Common causes leading to death in acutely infected cattle are shock and respiratory distress (Brown and Palmer, 1999).

3. *Babesia* life cycle: knowledge gaps, and opportunities for intervention

Babesia bovis and *B. bigemina*, exhibit a typical apicomplexan life cycle characterized by merogony, gametogony, and sporogony; have erythrocytes as the only single cell target in the bovine host; and are transovarially transmitted (Riek, 1966; Melhorn and Schein, 1984; Hunfeld et al., 2008; Chauvin et al., 2009).

The *Babesia* life cycle was recently reviewed in depth (Chauvin et al., 2009) however a brief revision of the life cycle will help to illustrate recent advances and some of the important knowledge gaps that remain. The known antigens briefly discussed here and currently presumed to be involved in at different stages of the cycle are also described in Table 1.

Both *B. bovis* and *B. bigemina* sporozoites enter the hosts with the saliva of the infected feeding tick larva (*B. bovis*) or nymph (*B. bigemina*). Sporozoites invade erythrocytes where they divide asexually by binary fission to become merozoites. Free and mature intraerythrocyte

B. bovis merozoites are shown in Fig. 1, Panels A and B, respectively. The mechanisms of erythrocyte invasion by sporozoites remain unknown, however, key *B. bovis* molecules that are required for erythrocyte invasion such as MSA-1, MSA-2, and RAP-1 (Table 1), are also expressed in surface of the sporozoites (Mosqueda et al., 2002a,b). Upon release, merozoites invade new erythrocytes where they transform into trophozoites (Fig. 1C) that divide by binary fission (merogony) to produce a pair of merozoites that perpetuate the cycle of erythrocyte invasion upon exiting the erythrocyte. Our understanding of the process of erythrocyte invasion by *B. bovis* merozoites remains incomplete and was reviewed by Yokoyama et al. (2006). A study using an *in vitro* culture invasion assay suggested that invasion of erythrocytes by *B. bovis* is likely Ca^{2+} dependent, and requires the function of an actin–myosin-based motor, thus following the target cell invasion pattern of other related apicomplexan such as *Plasmodium* and *Toxoplasma* parasites (Franssen et al., 2003; Carruthers and Boothroyd, 2007). Thus, invasion of erythrocytes by *Babesia* merozoites seems to involve an initial stage of erythrocyte-parasite recognition (Fig. 1B), probably using a sialic-acid dependent mechanism followed by attachment and re-orientation of the merozoites (Gaffar et al., 2003; Yokoyama et al., 2006). It was postulated that GPI-anchored, neutralization sensitive parasite proteins, members of the variable merozoite surface antigen (VMSA) that are expressed uniformly in the surface of *B. bovis* merozoites, are involved at the attachment stage of invasion (Hines et al., 1992; Jasmer et al., 1992a; Suarez et al., 2000; Florin-Christensen et al., 2002; Carcy et al., 2006; Yokoyama et al., 2006). The GPI anchored gp45 antigen, a surface-exposed 45 kDa variable protein that was able to induce protection against challenge with homologous strain was also identified in *B. bigemina* (McElwain et al., 1987, 1991; Fisher et al., 2001). In addition, the possible role of the surface exposed, conserved, and neutralization sensitive Bbo-MIC1 protein containing a putative sialic-acid binding domain in this process should also be explored further (Silva et al., 2010). As a result of re-orientation, the apical end of the merozoite comes in close contact with the surface of the erythrocyte. Similar to other apicomplexans, the apical end of the *Babesia* merozoites is equipped with organelles, such as micronemes, rhoptries, and spherical bodies that contribute with their secretions to the process of internalization of the parasites (Potgieter and Els, 1977; Sam-Yellowe, 1996; Yokoyama et al., 2006). Based on studies done on *Plasmodium* and *Toxoplasma*, it is hypothesized that during invasion, the microneme contents are secreted first followed by the contents of the rhoptries and the spherical bodies, an organelle resembling the dense granules in *Plasmodium* parasites (Mital and Ward, 2008; Carruthers et al., 1999). Importantly, *B. bovis* homologs of the AMA-1 (BbAMA-1) and TRAP (BbTRAP) (Gaffar et al., 2004a,b), and *B. bigemina* AMA-1 (Torina et al., 2010), that are known to be secreted by micronemes in *Babesia* related organisms such as *Plasmodium* and *Toxoplama*, have also been identified (Table 1). The exact functional role and the ligands for these molecules remain unknown, but because BbAMA-1 and BbTRAP contain neutralization sensitive epitopes, they are believed to play a significant role in invasion, and thus are being considered as subunit vaccine candidates. *B. bigemina* invasion studies suggested that the invading *Babesia* parasites form a short lived parasitophorous vacuole (PV) upon invasion that, in contrast to *Plasmodium*, appears to disappear shortly after internalization (Potgieter and Els, 1977; Yokoyama et al., 2006). It was suggested that the PV dissolves soon after internalization in *B. bovis* (Yokoyama et al., 2006), although the occurrence of a parasitophorous vacuole-based network or a parasite surface coat in *B. bovis*-infected erythrocytes was also proposed recently (Okamura et al., 2007). At least four *B. bovis* spherical body proteins, SBP-1, SBP-2, SBP-3, and SBP-4 have been so far identified (Jasmer et al., 1992b; Hines et al., 1995a,b; Dowling et al., 1996; Ruef et al., 2000; Yokoyama et al., 2006; Terkawi et al., 2011), but their biological roles remain unknown (Yokoyama et al., 2006). In addition, there are only two types of putative rhoptry *Babesia* proteins identified so far, the members of the RAP-1 family, identified in *B. bovis*, *B. bigemina*, and in other *Babesia* parasites (Suarez et al., 1991, 1993; Mishra et al., 1992; Dalrymple et al., 1993; Machado et al., 1993), and the *B. bovis* BboRhop68 (Baravalle et al., 2010). The RAP-1 molecules are expressed in merozoites, sporozoites and other asexual stages, are able to bind erythrocytes and contain neutralization sensitive B-cell epitopes, and have been a leading candidate for vaccine development (Yokoyama et al., 2002, 2006; Brown and Palmer, 1999; Norimine et al., 2003). However, their ligands and their exact roles in the invasion/egression mechanisms remain uncertain. The *B. bovis* RAP-1 proteins are encoded by two identical head-to-tail and tandemly oriented genes (Dalrymple et al., 1993; Suarez et al., 1998). In *B. bigemina*, the *rap-1* gene family is more complex, consisting of a total of 5 *rap-1a*, 5 *rap-1b,* and 1 *rap-1c* genes organized head-to-tail tandemly (Suarez et al., 2003). They are all transcribed but only the *rap-1a* genes appear to be translated. Yet, a novel RAP-1 related antigen (RRA), highly similar to *B. bigemina* RAP-1b, containing all the motifs that define the babesial RAP-1 proteins have also been recently identified in *B. bovis* (Suarez et al., 2011). RRA is expressed in minute amounts in merozoites, contains neutralization sensitive epitopes, it is highly conserved among distinct strains, and in contrast to RAP-1, is a subdominant antigen in infected cattle. Interestingly, the recently identified 68 kDa BboRhop68 was detected in intraerythrocytic trophozoites and merozoites but not in free merozoites suggesting that it is differentially expressed and may have a functional role immediately after *B. bovis* erythrocyte invasion, or erythrocyte egression by the mature merozoites (Baravalle et al., 2010). Thus far, the mechanism used by *Babesia* merozoites to egress from erythrocytes remains completely uncharacterized, and may also be a potential target for control.

Sexual reproduction of *Babesia* parasites starts when some intracellular trophozoites develop into gametocytes that are ingested by the ticks during feeding. The ingested gametocytes undergo several morphological changes in the midgut of the ticks (ray bodies) through undefined mechanisms to become competent for sexual reproduction, which requires the fusion of male and female gametes to generate zygotes (Melhorn and Schein, 1984; Riek, 1966). It is possible that, similar to *Plasmodium*, the sexual forms of

the parasites express sexual stage specific molecules, but they remain unidentified. Sexual stage proteins expressed in *Plasmodium* includes the 6-cys protein family, which includes the sexual stage antigen s48/45 domains (Pradel, 2007). Comparative genomic approaches allowed identification of a novel *B. bovis* gene family encoding proteins with similarities to the *Plasmodium* 6cys protein family, termed the *Bbo-6cys* gene family containing six genes termed Bbo-6cys-A to F (Table 1). The *Bbo-6cys* A to E genes are tandemly arranged as a cluster on chromosome 2 in the *B. bovis* genome, whereas the *Bbo-6cys* gene F is located in a distal region in the same chromosome (Silva et al., 2011). At a minimum the Bbo-cys6 E gene is expressed in blood stage parasites, and because it contains neutralization sensitive B-cell epitopes, it might be of functional relevance and possibly required for survival of the parasites, but the pattern of expression of the remaining genes of this family and their functional significance remains unknown and require further investigations.

The zygotes, that are likely diploid, invade digestive cells in the midgut of the ticks. Although it was reported that *B. bigemina* meiosis occurs in the midgut upon zygote infection (Mackenstedt et al., 1995), it remains unclear where and how meiotic division occurs for *B. bovis*. Zygotes then transform into kinetes (Fig. 1D) that can gain access to the hemolymph in the haemocoel of the tick vectors, where they invade the eggs in the tick's ovaries. This process allows transmission of *Babesia* parasites to the next generation of ticks, a mechanism known as transovarial transmission. Once in the larvae, the kinetes invade the cells in the salivary gland where they become sporozoites. An important expansion in the number of sporozoites occurs in the salivary glands through sporogony (Homer et al., 2000). Sporozoites are transmitted to the vertebrate hosts after at least 2 or 3 days following attachment of *B. bovis*-infected tick larva, whereas transmission occurs during tick nymph stages for *B. bigemina*. The events occurring in the *B. bovis* and *B. bigemina* sporozoites residing in the salivary gland of the *R.* (*B.*) *microplus* ticks leading to the formation of infective sporozoites, and their relationship with the tick feeding mechanisms are also not yet well understood.

In summary, an overview of the life of the *Babesia* life cycle suggests there are many opportunities for developing intervention strategies for improved control, but it also reveals the existence of many important knowledge gaps. Specifically, more research is needed on the differential expression and functions of parasite molecules required during the different stages of parasite development, the molecular mechanisms and signals involved in invasion of tick cells and erythrocytes, the mechanisms of egression, the molecular events involved in the transition of the parasites to sexual stages, the process of sexual reproduction in the tick, the occurrence of recombination during the sexual stage, the timing and mechanisms involved in the meiotic division, and the processes involved in the sporozoite maturation in salivary glands of the ticks. Also, understanding of the mechanisms of sexual reproduction of *Babesia* parasites, could lead to the development of novel control methods designed to block *B. bovis* transmission by interference with the development of the sexual stage.

4. *Babesia* causes acute and persistent infections in cattle

The prepatent period following inoculation of *B. bovis* by tick larva is generally between 6 and 12 days, with peak parasitemia and the manifestation of other clinical signs reached 3–5 days thereafter (Bock et al., 2004). Yet, *B. bovis* parasitemia remains low in acutely infected cattle likely due to the sequestration of the parasite in capillaries. The disease can be fulminant causing high mortality in adult and cattle, but less than one-year-old cattle are more resistant. Both *B. bovis* and *B. bigemina* have the ability to cause persistent infection in the face of strong immune responses in cattle surviving the acute disease. The mechanisms involved in persistence may differ between these two parasites. Whereas *B. bovis* merozoites residing in erythrocytes can be sequestered in blood capillaries (mainly cerebral and kidney), parasite sequestration has not been found to occur in *B. bigemina*. *B. bovis* infected erythrocytes that remain sequestered do not circulate in the blood and avoid phagocytosis by spleen macrophages, favoring establishment of persistent infections (Allred, 2001; Allred et al., 2000; Al-Khedery et al., 2006; Hutchings et al., 2007). *B. bovis* parasites are able to remodel the surface of the erythrocyte resulting in a change of its mechanical and adhesive properties to their advantage (reviewed by Gohil et al., 2010). Ridges formed on the surface of infected erythrocytes (O'Connor et al., 1999; O'Connor and Allred, 2000; Allred, 2000) were found to contain proteins encoded by the *B. bovis* variable erythrocyte surface antigen (*vesa*) gene family. These ridges play a key role in cytoadhesion of the parasitized erythrocytes to epithelial capillary cells. In addition, the VESA proteins can undergo rapid antigenic variation, that likely occurs through segmental gene conversion of the *vesa* multigene family (Al-Khedery and Allred, 2006; Brayton et al., 2007). Therefore, expression of vesa proteins may be central to *B. bovis* persistence by playing a dual role in adhesion and immune evasion leading to the facilitation of parasite survival in the face of the immune pressure of the hosts. The mechanisms involved in the persistence of *B. bigemina* parasites in cattle has been less explored, and it remains unknown whether expression of variable gene families also play a role in persistence, or if other adhesion phenotypes are required for parasite survival in the hostile milieu of the host's strong immune responses. Interestingly, *B. bigemina* erythrocytes uniquely display host's IgM molecules on their surface (Echaide et al., 1998). It remains unknown whether surface displayed IgM plays a role in adhesion, but together with its lack of tissue sequestration, this suggests that *B. bigemina* may use distinct persistence mechanisms than *B. bovis*.

Despite clearance by macrophages and strong antibody response acting against the parasite, a few infected erythrocytes remain in circulation in persistently infected bovines which assures the maintenance of a pathogen reservoir for ongoing transmission (Howell et al., 2007).

5. Current methods of control of bovine babesiosis

The negative impact of bovine babesiosis has in endemic areas, dictates the need for implementing control mea-

sures. A key factor is that cattle between 3 and 9 months of age do not manifest clinical babesiosis. Furthermore, animals in this age group can develop long term and strong immunity against homologous and heterologous parasite strains upon challenge. The resistance of young animals to the disease (Trueman and Blight, 1978; Goff et al., 2001; Zintl et al., 2005) is the basis of enzootic stability, defined as the state where the relationship between host, agent, vector and environment is such that clinical disease occurs rarely or not at all (Bock et al., 2004), and also for the success of the strategy of vaccination with attenuated *Babesia* parasites in areas at risk. In contrast, *Bos taurus* cattle older than one year, are usually highly susceptible to *Babesia,* including the vaccine strains (Trueman and Blight, 1978). The occurrence of serious outbreaks of babesiosis in endemic areas is usually related to the breakdown of enzootic stability (Smith et al., 2000). Transition toward endemic instability usually results in catastrophic consequences for the herds and can be caused by environmental, mainly climatic, and/or human factors, including inconsistent tick control programs that result in the reduction of the inoculation rates and low degrees of antibabesial immunity in the herds. Therefore, the use of sensitive diagnostic tests is important to determine the degree of immunity in a herd. This can be achieved by the use of novel standardized ELISA tests based on the detection of anti-RAP-1 antibodies (Goff et al., 2006, 2008) or antibodies against other immunodominant and conserved antigens (Bono et al., 2008; Terkawi et al., 2011).

Taking into consideration all of these factors, the control of bovine babesiosis can be accomplished either by tick management, immunization, anti-*Babesia* drugs, such as diminazen (Berenil) and imidocarb (Bock et al., 2004), or by a combination of these approaches. However, as selection of imidocarb-resistant *B. bovis* parasites can be induced experimentally (Rodriguez and Trees, 1996), it is conceivable that inappropriate use of the babesicides may lead to emergence of drug-resistant *Babesia* strains in the field (Zintl et al., 2003). Also, both imidocarb and berenil are associated with residue problems in the food chain (Zintl et al., 2003; Mdachi et al., 1995), and because *B. bovis* and *B. bigemina* vaccine strains might be more sensitive to babesicides, drug residues remaining in treated animals may also interfere with vaccination efforts (Combrink et al., 2002).

A widespread method used to diminish the impact of acute clinical disease, is vaccination with attenuated *Babesia* parasites.

Babesia parasites vary in their virulence, ranging from mildly to highly virulent and *Babesia* strains are polyclonal and often contain subpopulations of parasites that differ in virulence (Cowman et al., 1984; Nevils et al., 2000; Dalrymple et al., 1992). The nature of the factors that determine the virulent phenotype of *Babesia* parasites remains unknown and is currently being investigated. The *B. bovis* attenuated parasites used in vaccines are derived by serial and rapid passage of a virulent strain among 20–30 splenectomized steers. In contrast, *B. bigemina* attenuation is usually performed by slow passages of a virulent strain in spleen-intact steers (Callow et al., 1979; Shkap and Pipano, 2000). However, in both cases, the parasite populations resulting from the serial passages generally have an attenuated phenotype when inoculated into spleen-intact 3–9 month-old steers. Although several explanations have been proposed (Callow et al., 1979; Carson et al., 1990; Kahl et al., 1982, 1983), the mechanisms involved in attenuation through serial passage remain unknown. An ideal feature of the *Babesia* parasites that are used in vaccines is that they are not transmissible by ticks. Vaccine strains that are not infective for ticks have been reported such as the *B. bigemina* G vaccine strain (Bock et al., 2004) and a *B. bovis* vaccine strain produced in Argentina (Mangold et al., 1993). Nevertheless, at least in Australia, where vaccination has been extensively used for many years, vaccination did not seem to be able to introduce *Babesia* infections into previously uninfected areas (Bock et al., 2004). Production of live vaccines requires the amplification of the resulting attenuated parasites either in controlled bovine donors or in *in vitro* cultures (Callow et al., 1997). Live *Babesia* vaccines are usually safely administrated to young 3–9 months old steers, but older animals can still be susceptible to the vaccine strain and have a higher risk of succumbing to severe acute disease upon vaccination.

6. Searching for subunit vaccine targets

Live vaccines have many drawbacks such as the requirement of a cold chain, a short shelf life, and the potential for the transmission of concurrent pathogens and for reversion to virulence (Shkap and Pipano, 2000; Fish et al., 2008). Because of the shortcomings, there is still the need for additional research on the development of alternative safer and better defined live or subunit vaccines. The observations that: (i) cattle persistently infected with *Babesia* are generally resistant to re-infection with related strains, termed concomitant immunity (Brown et al., 2006); (ii) immunity following immunization with live, attenuated *B. bovis* vaccine is known to last after parasites have been eliminated by drug treatment (Brown et al., 2006; Dalgiesh, 1993); and (iii) immunization with killed parasites or parasite extracts can confer some level of protective immunity following homologous or heterologous strain challenge (Bock et al., 1992; Timms et al., 1984; Montenegro-James et al., 1985; Pattarroyo et al., 1995), suggested that developing alternative subunit vaccines is an achievable goal. Furthermore, immunization with purified native surface exposed proteins such as RAP-1a and GP45 (McElwain et al., 1991), and with purified rhoptries also resulted in variable degrees of protection against acute clinical disease upon challenge with virulent *B. bigemina* (Machado et al., 1999). Development of subunit vaccines requires a more refined understanding of the nature of the protective immune responses against *Babesia* parasites. Briefly, protective adaptive responses require γ-interferon producing CD4+T cells, and the production of opsonizing IgG2 and complement fixing IgG1 antibody (Brown and Palmer, 1999). In addition, the spleen and innate immunity in general play an important role both by helping to control the spread of infection in the hosts, and to prime the adaptive protective immune response. The importance of the spleen is evidenced by the increased susceptibility of splenectomized animals to *Babesia* infection. The roles of the innate system and our state of the art knowledge on the nature of

protective immune responses were extensively reviewed recently (Brown et al., 2006; Goff et al., 2010).

The search for protective antigens was initially focused on identification of surface exposed conserved *Babesia* proteins of presumed functional relevance, and later, on those that were able to stimulate CD+4 lymphocytes that secrete γ-interferon, and in other strategies, as reviewed recently (Brown et al., 2006). Briefly, rational approaches toward the development of blood stage *Babesia* subunit vaccines were focused initially on the identification of surface exposed, conserved, functionally relevant, and highly antigenic proteins, such as the members of the Rhoptry Associated Protein-1 (RAP-1) of *Babesia*. However, despite their ability to stimulate T-helper cell γ-interferon and IgG, testing of full size recombinant *B. bovis* RAP-1 or of RAP-1 fragments containing defined T-cell epitopes in bovine immunization experiments failed to confer significant protection upon challenge of experimentally immunized cattle with virulent *B. bovis* parasites (Norimine et al., 2003). Another vaccine candidate, the variable Merozoite Major Antigen-1 (MSA-1), expressed uniformly on the surface of the *B. bovis* merozoites was also tested in cattle immunization trials on the basis of the ability of anti-MSA-1 antibodies to strongly inhibit development of the parasite in *in vitro* neutralization assay (Hines et al., 1992; Suarez et al., 2000). However, recombinant MSA-1 also failed to provide protection upon challenge with *B. bovis* virulent strains (Hines et al., 1995a,b). It was reported that vaccination in Australia of cattle with a combination of three recombinant *B. bovis* antigens that included a 38-kDa cysteine-rich protein designated 12D3, a 60 kDa rhoptry protein designated T21B4 and also called Bv60 and rhoptry-associated protein 1 (RAP-1), and a high molecular weight antigen designated 11C5, confers some protection upon challenge, but this immunogen was not further developed (Wright et al., 1992). In a recent subunit vaccine trial, it was shown that immunization of steers with combined recombinant MSA-2c, MSA-1 and 12D3 was not able to prevent clinical symptoms upon challenge but the antigens were able to elicit an immunological response that was sufficient to protect steers from a mild virulent strain of *B. bovis* (Alvarez et al., 2010). In addition, other members of the VMSA family containing neutralization sensitive epitopes, such as MSA-2b should also be explored (Dominguez et al., 2010). In conclusion, relatively few blood stage antigens have been tested as vaccines, suggesting that new vaccine candidate antigens need to be identified, and many other new avenues of subunit vaccine development using the already available vaccine candidates remain yet to be explored. Thus far, subunit vaccine trials based on immunodominant antigens have failed to stimulate protective immunity (Brown et al., 2006). An interesting emerging approach is the identification of subdominant, rather than immunodominant, antigens for subunit vaccine development. Subdominant antigens may be better vaccine candidates because antigens against which parasites allowed the host to mount an immune response (immunodominant) are likely unimportant for the survival of the organism (Brown et al., 2006). The recently identified subdominant *B. bovis* RRA (Suarez et al., 2011) could be an appropriate antigen for testing this emerging vaccine approach.

7. Perspectives for developing new preventive and therapeutic interventions against *Babesia* parasites

At least two major recent research developments that, in conjunction with the availability of *Babesia* culture systems and novel genomic, transcriptomic and proteomic techniques, have great potential to impact vaccine development are: (a) the complete genome sequence of *Babesia* parasites; and, (b) the availability of novel functional genomics tools, including a transfection system for *B. bovis*. In addition, other important recent advances on the characterization of the mechanisms of antigenic variation, the adhesion properties of infected erythrocytes, the mechanisms of cell invasion were reviewed in deep elsewhere (Barbet, 2009; Allred and Al-Khedery, 2006; Gohil et al., 2010; Hutchings et al., 2007; Yokoyama et al., 2006).

7.1. Availability of genome data

Availability of the genome sequence of *Babesia* spp. has improved our understanding of the biology of the parasite. Full genome sequences greatly accelerated the identification of novel vaccine and pharmacological intervention targets. The ~8.2 Mbp *B. bovis* genome contains at least 3671 protein coding nuclear genes distributed among four chromosomes (Brayton et al., 2007). The largest gene family found in the genome encodes the polymorphic variant erythrocyte surface antigen protein (*vesa*). Full structural characterization of the *vesa* genes coupled with the identification of the molecular basis and the structural determinants of the mechanisms involved in cytoadhesion can facilitate the development of a vaccine strategy aimed at blocking cell adhesion. Full genome analysis also has identified novel gene families encoding other proteins of potential functional significance such as the *smorf* gene family (Brayton et al., 2007; Lau, 2009).

Easily available comparative genomic tools, such as Blast searches, also facilitate identification of candidate genes that encode for proteins of functional relevance or are known to be targets of protective immune responses in other fully sequenced related apicomplexans, such as the more studied *Plasmodium* and *Theileria* parasites. This strategy allowed recent identification of previously unknown and un-annotated genes and genes families that have the potential for vaccine and drug development (Table 1), and for developing research leading to a better understanding of the biology of these parasites (Suarez et al., 2011; Silva et al., 2010, 2011; Baravalle et al., 2010). For example, comparative genomics led to the identification of p67, a *Babesia bovis* gene syntenic to *Theileria parva* p67 that is expressed in blood and tick stage parasites (Freeman et al., 2010) (Table 1). In addition, the recently characterized papain-like cysteine proteases involved in important metabolic pathways, are candidates for both vaccine and drug development (Mesplet et al., 2010). Additionally, genome data mining has allowed for the development of tandem repeat-based multilocus typing systems used to differentiate *B. bovis* geographic isolates (Perez-Llaneza

et al., 2010; Simuunza et al., 2011a,b), and the discovery of differential transcription of rRNA genes in *B. bovis* parasites in distinct environmental conditions (Laughery et al., 2009).

Interestingly these approaches, combined with structural chemistry studies, also proved to be essential in the identification of free GPI molecules and the genes encoding for enzymes involved in the assembly of GPI anchored proteins in the *B. bovis* genome (Rodríguez et al., 2010). Inhibition of the GPI-pathway using mannosamine in *in vitro* cultures resulted in abrogation in the growth of *B. bovis*, suggesting that this pathway is essential for survival of the parasites (Rodríguez et al., 2010). Therefore, these findings suggest that further studies on this newly identified metabolic pathway might lead to the discovery of novel drugs or vaccines that might be effective to control *Babesia* parasites.

The *B. bovis* genome sequence also facilitated the characterization of the *B. bovis* expression profiles. This approach can facilitate the identification of candidate vaccine antigens, help to characterize the profile of expression at different stages, and overall, improve our understanding of the biology of the parasite (Lau et al., 2007).

Taken together, these newly discovered genes and sequences exemplify the potential impact of the use of the information contained in the *B. bovis* genome. Genome data, complemented with the characterization of protective immune responses and the analysis of the structure and pattern of expression of genes encoding for functionally relevant proteins facilitates research on new approaches for developing subunit vaccines, new drugs, and our improved understanding of the biology of *Babesia* parasites. In addition to the genome data, the role of epigenetic regulation in *Babesia*, that so far remains unexplored, may also help us to better understand expression of certain phenotypes in these parasites.

7.2. Use of functional genomics tools in Babesia research

More than half of the known *B. bovis* genes remain un-annotated and the functional role and biological relevance of most, either annotated or un-annotated genes, is still unknown. The use of transcriptomic, proteomic and transfection approaches, in combination with other techniques, will be needed for the identification of genes that are differentially expressed in distinct stages of the parasite life cycle. In addition, transfection methods, which allow the incorporation and expression of foreign DNA into *Babesia*, are also useful for characterizing gene function, for studying mechanisms of gene regulation by using gene knock-out and supplementation strategies, and for vaccine development. A recent review described the development and potential applications of *Babesia* transient and stable transfection systems in more depth (Suarez and McElwain, 2010). Briefly, the transient transfection system has potential to be used for experiments requiring short term expression of the gene of interest, which in general are selectable markers (i.e. blasticin-gfp fusion gene) and/or reporter genes (i.e. luciferase, GFP) (Suarez et al., 2004, 2006; Suarez and McElwain, 2008, 2009, 2010). For instance, transient expression systems can be used for the characterization of signal peptides, to study trafficking pathways, or to characterize mechanisms of secretion used by the parasite. These studies could be performed by determining cellular localization of fluorescent molecular reporters, such as GFP, fused to the signal peptides under investigation. In contrast, stable transfection system requires more complex plasmid constructs and the obligatory use of a selectable marker. Recent experiments with stable transfection constructs were designed for integration into the elongation factor-1α (*ef-1α*) locus of the *B. bovis* parasite by homologous recombination (Suarez and McElwain, 2009). The *ef-1α* locus has an identical organization in both, *B. bovis* and *Plasmodium* parasites consisting of two identical head to head, tandemly arranged genes separated by intergenic regions containing strong and constitutive promoters (Vinkenoog et al., 1998; Suarez et al., 2006). Research performed in *Plasmodium* demonstrated that replacing one of two *ef-1α* genes present in malaria parasites by homologous recombination using transfection techniques would not greatly affect parasite viability (Janse et al., 2003). These observations provided rationale for the use of the *ef-1α* locus as a target of integration for the development of a *B. bovis* stable transfection system (Suarez and McElwain, 2010; Janse et al., 2003). The successfully transfected parasites were selected with antibiotic blasticidin, and they were able to express abundant and intracellular, cytoplasmic, green fluorescent (GFP-BSD) protein (Fig. 2A). Stable transfection systems have a wide range of possible application in vaccine research and development including: (1) helping to define virulence factors by gene knock-out and complementation techniques; (2) a tool aiding the functional characterization of genes; (3) the production of viable and attenuated *Babesia* strains that are deficient in known virulence factors; (4) introducing antigenic molecular markers that may help to discriminate vaccinated from naturally infected animals; (5) producing *Babesia* strains that express protective tick antigens, or that over-express differentially expressed or subdominant *Babesia* antigens. Current research efforts are focused on the application of transfection as a delivery system of foreign antigens, such as protective tick antigens with the goal of achieving dual vaccines that can protect cattle against clinical babesiosis and also interfere with transmission of the parasite by targeting protective tick antigens and *B. bovis* antigens that are exclusively or highly expressed in tick stages of the parasite.

8. Bovine anaplasmosis

Anaplasma marginale is the most prevalent tick-borne pathogen of cattle worldwide with endemic regions in North, Central, and South America, as well as Africa Asia, and Australia (Brayton et al., 2009). Ixodid ticks are the biological vector of *A. marginale*, while mechanical transmission can occur through fly bites and reuse of needles (Sonenshine, 1991). Once an animal is exposed to this pathogen acute infection develops, which is characterized by fever, high levels of bacteremia ($\geq 10^7$ bacterial/ml of blood), anemia, weakness, reduced growth and milk production, abortion, and in some cases, death (Kocan et al., 2010). Given time the acute infection is controlled

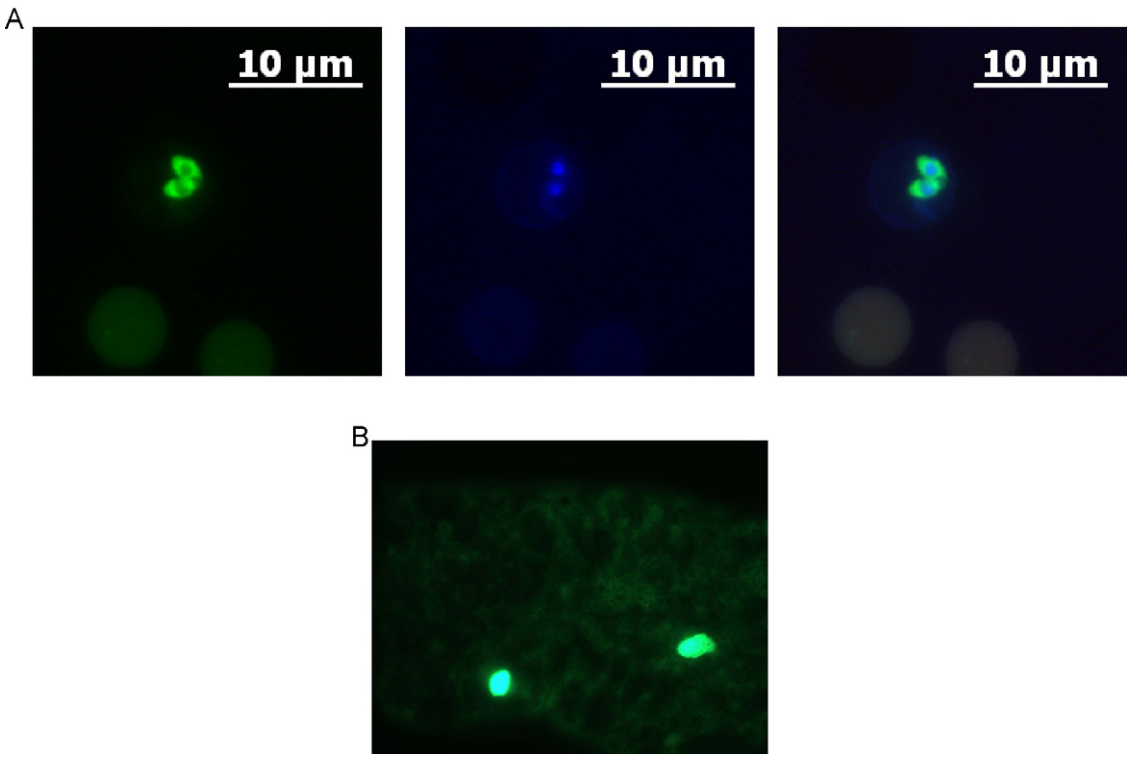

Fig. 2. Expression of green fluorescent protein (GFP) in transformed parasites as detected by epifluorescence microscopy. (A) *Babesia bovis* transformed with the *gfp-bsd* gene (parasite line 1-2-124) expressed from the ef-1α locus of *B. bovis* (Suarez and McElwain, 2009). Localization of the expressed chimeric protein was enhanced in formaldehyde-fixed cultured cells using an anti-GFP antibody and Alexa Fluor® 488 conjugated secondary antibody. Expression of chimeric protein (green) is evident within the cytoplasm of two intracellular merozoites whose nuclei have also been stained by DAPI (blue). Two autofluorescent non-infected cultured red blood cells are also visible (left panel = gfp immunofluorescence, middle panel = DAPI, right panel = merged images). (B) Green fluorescent colonies of the St. Maries strain of *A. marginale* transformed with *gfp* in the midgut of a *Dermacentor andersoni* adult male tick after 7 days of acquisition feeding.

and persistent infection develops, which is thought to last for the life of the animal (Smith et al., 1989). During persistent infection, *A. marginale* is maintained below microscopically detectable levels which fluctuate between 10^3 and 10^6 *A. marginale*/ml of blood, and overt clinical signs are generally absent (Eriks et al., 1993; French et al., 1998; Kieser et al., 1990). Transovarial transmission of *A. marginale* from one tick generation to the next does not occur, thus the establishment of persistent infection within the bovine host is essential for ongoing transmission of this pathogen.

9. Available control methods

Currently, the tools available to prevent anaplasmosis are limited, and rely on the use of a live vaccine, antibiotic treatment, and the maintenance of endemic stability within a herd. The live vaccine, discussed in the next section, is not available in all parts of the world and antibiotic treatment of clinically affected animals is difficult and expensive in extensive animal rearing systems. Prevention of infection relies solely on tick control in the face of increasing acaricide resistance among tick populations (George et al., 2004; Rosario-Cruz et al., 2009). While antibiotic treatment is effective in decreasing bacterial numbers and ameliorating disease, the efficacy in clearing infection and thus preventing the establishment of a pathogen reservoir is variable. Recently, treatment of persistently infected steers that with oral chlortetracycline at doses between 4.4 and 22 mg/kg per day for 80 days cleared *A. marginale* infection (Reinbold et al., 2010). The absence of infectious organisms was demonstrated by PCR by day 49 of the treatment. Sub-inoculation of blood into splenectomized animals was used to confirm complete clearance of the organism. Discouragingly, animals cleared of infection through treatment were then re-infected with the original strain (Reinbold et al., 2010). The results of treatment trials have been variable. In a second study, persistently infected animals treated with 22 mg/kg of oxytetracycline intravenously every 24 h for 5 days failed to clear the infection (Coetzee et al., 2005). Additionally, long treatment of animals with antibiotics on a large scale is becoming less acceptable as antibiotic resistance rises in both human and animal pathogens. Given the significance of the disease burden, research started with the discovery of *A. marginale* by Theiler (1910) in South Africa is being continued today in order to identify new means of disease control and prevention. There are two likely targets of intervention: (1) at the level of bovine immune system by induction of protective immunity against *A. marginale* through vaccination and; (2)

less conventionally at the level of the tick, by preventing tick colonization and transmission of *A. marginale*.

10. Identification of *A. marginale* vaccine targets

In contrast to natural exposure, which often results in acutely high levels of bacteremia and anemia and long-term persistence; immunization results in a significant reduction in bacteremia and anemia in nearly all animals and depending upon the immunogen, protection against infection in 40–70% of immunized animals, providing a rationale for vaccine development (Brown et al., 1998; Tebele et al., 1991; Noh et al., 2008). The mechanisms of immune control of *A. marginale* are not completely understood. However, protection against challenge associates with IgG_2 directed toward outer membrane proteins (Brown et al., 1998; Palmer et al., 1999; Tebele et al., 1991). Two effective immunogens have been described; the first is *A. marginale* subsp. *centrale*, which is used as a live vaccine. The second is composed of outer membrane proteins of *A. marginale* and has only been used experimentally (Bock and de Vos, 2001; Pipano, 1995; Tebele et al., 1991). *A. marginale* subsp. *centrale* was isolated in S. Africa in 1911 (Theiler, 1911). Soon thereafter protective immunity against virulent *A. marginale* challenge was demonstrated in animals immunized with this organism (Theiler, 1912). *A. marginale* subsp. *centrale*, while still used as a live vaccine in many parts of the world has a high production cost, and carries the risk of vaccine-induced disease as well as transmission of known and unknowns pathogens. Additionally, immunity induced by live vaccination with *A. marginale* subsp. *centrale* is not uniform against all strains and outbreaks have been reported in immunized populations (Brizuela et al., 1998; Turton et al., 1998). However, the overall efficacy of this vaccine demonstrates that protection against heterologous challenge in terms of disease prevention is possible (Shkap et al., 2002). The protective outer membrane immunogen is derived from *A. marginale* isolated from erythrocytes. After lysis, the outer membrane fraction of the isolated bacteria is concentrated using a sucrose gradient. Consequently this immunogen is complex, laborious to formulate, and thus is not appropriate for mass production (Lopez et al., 2005; Tebele et al., 1991). Additionally, cross-protection among strains using the outer membrane immunogen has not been widely tested. Despite these constraints, these two immunogens coupled with the complete genome sequence of several strains of *A. marginale* serve as the foundation to identify the antigens relevant for induction of broad, cross-protective immunity.

The purified outer membrane of *A. marginale* which induces protective immunity is composed of over 20 different proteins. The major surface proteins (Msps), including Msp1, Msp2, Msp3, Msp4, and Msp5, which are all present in the outer membrane immunogen, were the first to be identified based on their abundance and their ability to induce large amounts of antibody (Palmer and McGuire, 1984). However, immunization with native, purified or recombinant Msps generally leads to poor protection (Palmer and McElwain, 1995). Of particular interest are Msp2 and Msp3 in part, because large amounts of antibody are directed toward these abundant, hypervariable outer membrane proteins. Msp2 and Msp3, importantly, are involved in immune evasion and establishment of persistent infection though a mechanism of antigenic variation. This process has been reviewed elsewhere (Palmer et al., 2009). Briefly, Msp2 is expressed from a single expression site, and is composed of a central hypervariable region that is flanked by highly conserved regions. Msp3 is thought to function similarly, though has not been examined as extensively (Brayton et al., 2003; Futse et al., 2009; Meeus et al., 2003). During infection within the host, the variation in the expressed version of Msp2 is generated by gene conversion in which one of multiple *msp2* donor alleles is recombined into a single, operon-linked expression site (Barbet et al., 2000; Brayton et al., 2001, 2002). Clearance of a specific *A. marginale* Msp2 variant is accompanied by development of an antibody response directed toward that variant, indicating that Msp2, likely in combination with Msp3, is responsible for the ability of *A. marginale* to evade the bovine immune response. Thus, Msp2 and Msp3 have seemed likely vaccine targets. Experiments using both Msp2 and Msp3 as an immunogen have not been conducted. However, immunization with native purified Msp2 containing a wide variety of variants did not confer protection to cattle challenged with *A. marginale* expressing the same Msp2 variants as in the immunogen (Abbott et al., 2005). Additionally, antibody directed toward Msp2 correlated with control of bacteremia infection, but not correlate with protection due to immunization (Noh et al., 2010b). Thus, the focus in identification of vaccine candidates has shifted toward the identification of subdominant antigens that confer protective immunity. This approach is rational as subdominant antigens tend to be less variable. The utility of this approach has been confirmed. For example, subdominant antigens have been identified that induce neutralizing antibody to a variety of strains of the influenza virus (Ekiert et al., 2009; Kubota-Koketsu et al., 2009).

Through genomic and proteomic approaches, the vaccine-relevant genome has been narrowed, and in particular, subdominant antigens have been identified using experiments linking vaccination/challenge trials with detailed proteomic analysis of the protective immunogen. In one approach, the complex outer membrane protein immunogen was separated into individual and small groups of components using 2-dimensional gel electrophoresis. The immunoreactive proteins were then identified using western blotting with sera from cattle immunized with the protective *A. marginale* outer membrane proteins. The immunoreactive proteins were excised and subjected to liquid chromatography followed by mass spectrometry for definitive identification by mapping to the annotated genome (Lopez et al., 2005). This approach identified 24 immunodominant and subdominant antigens which induced IgG_2 production in the response to immunization. These included the previously characterized major outer membrane proteins Msp2, Msp3, and Msp5, 21 newly described antigenic proteins, and multiple housekeeping genes, which likely reflect the heterogeneous nature of the outer membrane protein immunogen.

To further narrow the potential vaccine targets and more closely link protective immunity with antigen identification, two approaches were taken. In the first approach,

intact *A. marginale* were isolated and treated with a membrane impermeable cross-linking reagent, which resulted in covalent linkage of a group of surface exposed outer membrane proteins. After purification, the protein complex was used to immunize cattle, and was analyzed by mass spectroscopy (Noh et al., 2008). This protein complex included only a subset of the complex outer membrane immunogen, but remained capable of the same significant level of protection (Noh et al., 2008). Based on the proteomic analysis, this protective complex contained 11 outer membrane proteins: Omp1, Omp7–9, Msp1a, Msp2, Msp3, Msp4, OpAG2, Am779, and the peptidoglycan-associated Am854 (Noh et al., 2008).

In the second set of experiments IgG_2 from *A. marginale* subsp. *centrale* immunized cattle was used to probe blots of *A. marginale* outer membranes separated in two dimensions. The rational of these experiments was to identify antigens in *A. marginale* that are conserved in the protective vaccine strain (Agnes et al., 2011). In addition to Msp2 and Msp3, outer membrane proteins Omp7–9, 11, 13, and 14, Am779, and AM854 were recognized. Excluding the hypervariable Msp2 and Msp3, conservation between the other antigenic surface proteins of *A. marginale* and *A. marginale* subsp. *centrale* ranged from 60 to 84%, indicating that a high degree of overall identity is not required to maintain antigenic cross-reactivity. After excluding Msp2, and Msp3, the two studies identified five outer membrane proteins in common, Omps 7–9, Am779 and Am854, indicating that pursuit of non-variable surface proteins may be rewarding in vaccine development.

There is a gap in knowledge concerning the variability of the outer membrane protein repertoire among populations of *A. marginale* from geographically diverse regions. However, there is strong evidence suggesting that a subset of the vaccine candidates may be conserved and thus development of a widely cross-protective vaccine is possible (Junior et al., 2010). For example, Omp7 one of the vaccine candidates, has high % identity (86–99%) when comparing the predominantly North American strains (Agnes et al., 2011). This identity drops to 65–72% when comparing Omp7 from a Brazilian strain to the St. Maries and Florida strains, respectively (Junior et al., 2010). In contrast, many other outer membrane proteins have a high % identity (92–100%) when comparing two N. American strains to a Brazilian strains.

11. Mechanisms of tick transmission

A. marginale is an obligate intracellular pathogen, which displays marked host cell specificity. The transition from the bovine host to the tick is a dramatic shift in the host cell environment from the non-nucleated, metabolically quiescent bovine erythrocyte to the metabolically active, phagocytic and digestive tick midgut epithelial cell. The pathogen must then traverse the hemolymph evading the tick immune system in order to colonize the salivary glands. Upon ingestion of a second blood meal, *A. marginale* is transmitted to a new mammalian host. Understanding the molecular foundation of tick colonization and transmission presents largely unexplored opportunities for control of this pathogen. Little is known about either the pathogen or vector molecules required for *A. marginale* to successfully colonization and be transmitted from the tick.

Many species of ixodid ticks serve as competent vectors of *A. marginale* (Kocan et al., 2010; Sonenshine, 1991). Most notable are members of the genus *Rhipicephalus* due to their wide geographic distribution, overall debilitating effects when present in high numbers on a host, and ability to transmit *Babesia* spp. and *A. marginale*. Much of the research concerning tick transmission of *A. marginale* has been done using *Dermacentor* spp., a primary vector of *A. marginale* in the United States, in part because of the size and hardiness of this tick in laboratory conditions. When comparing the ability of *D. andersoni* and *R. microplus* to transmit the Puerto Rico and Florida strains of *A. marginale*, there were no differences in vector competence in terms of % infected ticks and pathogen transmission (Scoles et al., 2007). Experiments using quantitative PCR to determine the pathogen load in the tick organs have also been done. In these experiments, the ability of *D. andersoni* and *R. microplus* to transmit the St. Maries strain of *A. marginale*, isolated in the north western United States, and the Puerto Rico strain were compared (Futse et al., 2003). Both species of ticks were similarly able to acquire and transmit both strains of *A. marginale* and both had similar infection rates (% infected ticks). The Puerto Rico strain replicated to higher levels in the salivary glands of *R. microplus* than *D. andersoni* during transmission feeding, suggesting some degree of adaptation between the pathogen strain and its available vector. While these two tick species perform similarly in laboratory conditions, the role they play in the epidemiology of disease is unknown. In temperate regions, where *Dermacentor* spp. are the primary vector, the prevalence of *A. marginale* within infected herds tends to be relatively low. For example, 15% of animals in feedlots in Iowa, located in the Midwestern United States, were seropositive (Coetzee et al., 2010). In contrast, in tropical regions, the prevalence tends to be quite high, from 37% in Costa Rica (Oliveira et al., 2011) to 58% in the Paraná state of Brazil (Marana et al., 2009) and Mbeere district of Kenya (Gachohi et al., 2010), and up to 83% in some regions of Zambia (Simuunza et al., 2011a,b). There are likely many reasons for these differences, one of which may be the tick vector.

A. marginale strains with clearly different transmission phenotypes have been characterized and serve as useful tools to identify the genetic requirements of tick transmission. For example, the St. Maries strain, which was isolated from an animal in the Northwestern United States suffering from severe anaplasmosis (PCV = 7%), is readily transmitted, with $\leq$10 *Dermacentor andersoni* ticks being sufficient for consistent transmission (Eriks et al., 1994; Scoles et al., 2005). In contrast, *A. marginale* subsp. *centrale* has a low tick transmission efficiency phenotype. This organism infected fewer ticks, and replicated to significantly lower levels in the tick midgut and salivary gland as compared to the St. Maries strain (Ueti et al., 2009). Based on these quantitative differences as compared to the high transmission phenotype of the St. Maries strain, transmission of *A. marginale* subsp. *centrale* is estimated to require >350 *D. andersoni*. This is supported by replicate trials in which 70–250 adult male *D. andersoni* ticks

fed during either acute or persistent infection were unable to transmit *A. marginale* subsp. *centrale* (Agnes et al., 2010; Galletti et al., 2009; Ueti et al., 2007, 2009) while successful transmission was observed with 425 adult male *D. andersoni* (Ueti et al., 2009). Attempts to transmit *A. marginale* subsp. *centrale* using *Hyalomma excavatum*, *Rhipicephalus sanguineus*, and *B. annulatus* ticks all failed. However, less than 200 ticks, well below the estimated number required for transmission, were used in all cases (Shkap et al., 2009). At the far end of the spectrum are the apparently non-tick transmissible strains. Despite various attempts with several tick species, the Florida and Mississipppi strains have not been successfully tick transmitted (Smith et al., 1986; Ueti et al., 2007). It has been hypothesized that these observed differences in tick transmission efficiency will be reflected in genetic differences among *A. marginale* strains. More specifically, phenotypically distinct strains may differ in transmission efficiency due to strain-specific gene content such that the presence or absence of a specific gene confers a tick transmission phenotype. Alternatively, a polymorphism in a gene may alter the function of the product thus leading to either increased or decreased transmission efficiency. A third possibility is that differences in regulatory regions may lead to increased or decreased expression in genes which are common to both highly and poorly transmitted strains (Agnes et al., 2010). While not all of these possibilities have been addressed experimentally, both comparative genomic and proteomic approaches have been used to identify the molecular requirements of tick transmission.

First, a comparative genomics approach was used to link specific gene content with the tick transmission phenotype. The genomes of the efficiently tick transmitted Virginia, Puerto Rico, and St. Maries strains were compared to the non-tick transmitted Mississippi and Florida strains (Dark et al., 2009). Specific genes associated with transmission phenotype were not identified (Dark et al., 2009). Thus, the differences in transmission phenotype are not due to strain-specific gene content, but more likely due to differences in shared genetic elements, either in coding or regulatory regions.

Two proteomics based approaches have been used to identify proteins up-regulated during tick colonization (Ramabu et al., 2010; Noh et al., 2008). The first approach involved comparing cross-linked surface exposed protein complexes between *A. marginale* isolated from erythrocytes and ISE6 tick cells. Overall the variety of proteins expressed on the *A. marginale* colonizing the tick cells was reduced to five as compared to fifteen identified on A. *marginale* from bovine erythrocytes. Four proteins, Msp2, Msp3, Msp4, and Am854, a peptidoglycan-associated protein, were expressed in common. Am778 was the only protein to be uniquely expressed on the *A. marginale* isolated from tick cells. These results were non-quantitative, and were not verified in *A. marginale* colonizing the tick vector. In a second quantitative approach, proteins from *A. marginale*-infected and uninfected tick cells and infected bovine erythrocytes were separated using two-dimensional gel electrophoresis and stained (Ramabu et al., 2010). Spots which were unique to infected tick cells were submitted for mass spectrometric analysis. From those spots, 15 *A. marginale* proteins, all annotated as hypothetical were identified, including the previously identified Am778 as well as Am638, an ankyrin-repeat containing protein. Additionally, many metabolic and housekeeping genes were identified, including, but not limited to dnaK, groEL, rpA. Two type 4 secretion system proteins were also identified, VirB10 and VirB11. Confirmation of the up-regulation of a subset of these proteins, including Am470, Am410, and Am829 in tick cells as compared to bovine erythrocytes was done using western blotting and densitometry. Expression of these proteins in *D. andersoni* midguts and salivary glands was verified using immunohistochemistry. Of particular interest is the ankyrin-repeat containing protein, Am638. Ankyrin repeats are highly conserved motifs that are thought to mediate protein–protein interaction and often localize to the host cell nucleus (Pan et al., 2008). Ankyrin repeat containing proteins from *E. chafeensis* and *A. phagocytophilum*, pathogens related to *A. marginale*, have been shown to traffic to the mammalian host cell nucleus (Garcia-Garcia et al., 2009a,b; Zhu et al., 2009). Because *A. marginale* resides in non-nucleated bovine erythrocytes during mammalian infection, proteins which localize to the host cell nucleus would only be relevant during tick infection. It is unknown how these proteins actually function during *A. marginale* infection.

These proteomic approaches rely on differential expression of the respective gene products to infer functional significance. While it is commonly hypothesized that proteins required for tick colonization are up-regulated during tick colonization, additional constitutively expressed proteins may also be required. For example, many *Mycobacterium tuberculosis* genes required for survival within the macrophage are not differentially regulated within the macrophage, but rather are constitutively expressed (Rengarajan et al., 2005). The genetic tools needed to test functional requirement of both up-regulated and constitutively expressed proteins are in the early stages of development for *Anaplasma* spp. (Felsheim et al., 2006). The St. Maries strain of *A. marginale* has been successfully transformed with a *green fluorescent protein* (*gfp*) gene (Felsheim et al., 2010). The tick transmissibility, and ability to establish persistent infection in the bovine was recently demonstrated thus establishing the utility of this transformant as a model organism (Noh et al., 2010a). Further development of techniques to create targeted gene knockouts, and mutant libraries will be essential for moving beyond association to identification of gene function.

12. Closing arguments

While developing *Babesia* subunit vaccines might still be an achievable goal, new tools will also allow for the production of improved genetically modified live vaccines to be developed using the framework of the current effective live vaccines. Thus, future improved live *Babesia* vaccines based on genetically attenuated organisms that can also function as a delivery system for protective antigens expressed in stages other than erythrocytic and/or from the tick vectors could be produced using transfection methods.

In addition, the two veins of anaplasmosis research summarized above are currently being mined with the goal of developing effective tools to either induce immunity in the bovine host through traditional methods of vaccination or to prevent transmission of *A. marginale* through, as yet, undiscovered methods of blocking tick transmission. Rapid progress in vaccine development will require a better understanding of the correlates of immunity of *A. marginale*, which can then be used to develop *in vitro* techniques to rapidly screen vaccine candidates. The development of tick transmission blocking tools will require the ability to identify gene function, in particular to identify genes required for tick colonization and transmission. Consequently, the ability to create targeted gene knock-outs and whole genome mutant libraries of *A. marginale* will greatly facilitate this venture.

Overall, the use of genome data in conjunction with novel transfection and genomic related approaches will be instrumental for closing our current knowledge gaps toward a better understanding of the biology of these organisms and for formulating new vaccines and other therapeutic interventions. Together with a better understanding of the protective immune responses, these new experimental approaches will likely be the keys leading to the developing of improved methods of control of these devastating cattle diseases.

Conflict of interest statement

The authors state no conflict of interests.

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